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The origin and early development of wheat glutenin particles

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ABSTRACT

Breadmaking quality is strongly related to the glutenin macropolymer (GMP) fraction. Don and co-workers [Don, C., Lichtendonk, W.J., Plijter, J.J., Hamer, R.J., 2003a. Glutenin macropolymer: a gel formed by particles. Journal of Cereal Science 37, 1–7] showed that GMP consists of spherical glutenin particles and suggested that these originate from the protein bodies (PBs) observed in developing grain. We have tested this hypothesis by systematically comparing SDS-soluble and SDS-insoluble protein fractions from both PBs and flour. These preparations were analysed at the molecular, oligomer, particle and microscopic levels. Comparison of PBs isolated from immature seeds with glutenin particles isolated from mature seeds revealed strong similarities in protein composition and the presence of large glutenin oligomers. However, the glutenin particles from mature wheat were significantly larger than PBs. We suggest that PBs are the building blocks for the formation of much larger glutenin particles which are formed during the desiccation phase of kernel development.

1. Introduction

Gluten polymers from wheat are among the largest and most complex protein networks in nature with MWs of more than 10 million (Wieser, 2007). The differences in breadmaking quality between flours of different wheat varieties also parallel differences in a gluten protein fraction called glutenin macropolymer (GMP) (Graveland, 1984; Graveland et al., 1982; Weegels et al., 1996, 1997). GMP is the glutenin fraction which is insoluble in various solvents (SDS or acetic acid) (Weegels et al., 1996, 1997) and consists of spherical glutenin particles (Don et al., 2003). These glutenin particles also vary in size, and this variation correlates with a key technological quality parameter: dough mixing properties (Don et al., 2005). The genetic background and growing conditions also affect the quality of GMP, the glutenin particle size and, consequently, flour quality, but the mechanism behind this effect is not known (Don et al., 2005). However, Don and co-workers (2003) suggested that the glutenin particles in GMP are related in origin to the PBs observed in developing wheat endosperm.

The wheat gluten proteins can be classified into three groups on the basis of their structural and evolutionary relationships. These are sulphur-rich (S-rich), sulphur-poor (S-poor) and high molecular weight (HMW) groups, with a number of subgroups within the S-rich and S-poor groups. However, this classification does not correspond directly to the polymeric and monomeric fractions in the wheat kernel (glutenins and gliadins, respectively) (Köhler et al., 1993), with the HMW subunits and the S-rich LMW subunits being the main contributors to glutenin particles.

Wheat seed storage proteins are synthesised in developing grain and are initially deposited in discrete protein bodies (PBs) (Shewry and Halford, 2002). All gluten proteins are initially synthesised on the rough ER and co-translationally translocated into the lumen. However, it appears that some components, probably mainly gliadins, are transported via the Golgi to the protein storage vacuole whereas others, probably mainly glutenins, are deposited within the lumen of the ER to form a second population of ER-derived protein bodies (Kim et al., 1988; Rubin et al., 1992). It has also been suggested that these ER-derived protein bodies are subsequently absorbed by protein storage vacuoles in a process analogous to autophagy (Galili, 1997; Shewry, 1999; Shewry and Halford, 2002). This leads to the formation of an apparently continuous gluten

Abbreviations: CSLM, confocal laser scanning microscopy; DAF, days after flowering; ER, endoplasmic reticulum; FITC, fluorescent protein label; FPLC, fast protein liquid chromatography; GMP, glutenin macropolymer; HMW, high molecular weight; HPLC, high performance liquid chromatography; LMW, low molecular weight; PAGE, polyacrylamide gel electrophoresis; PBs, protein bodies; SDS, sodium dodecyl sulphate; SE, size exclusion.

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protein matrix in the cells of the mature grain (Parker, 1980). The PBs in developing grains also contain dark-staining inclusions of the globulin storage protein triticin (Bechtel et al., 1991).

The accumulation of wheat seed storage protein commences as early as seven days after flowering (DAF) and ends at the beginning of the desiccation phase. In contrast, a close correlation was found between the accumulation of the GMP and the rapid loss of water during desiccation (Carceller and Aussenac, 1999, 2001). The percentage of SDS-insoluble polymers as a proportion of total polymers can increase from less than 10% at the end of kernel ripening to 50% in as little as 10 days, although the synthesis of gluten proteins is completed before this occurs (Carceller and Aussenac, 1999). Since glutenin insolubility is related to aggregate size, this indicates a lower degree of aggregation within PBs from immature wheat grain compared to within the gluten matrix of the mature wheat grain.

It has been recently suggested that the glutenin particles isolated from mature wheat flour and observed as spherical shaped particles using confocal laser scanning microscopy (CLSM) originate from the individual PBs as observed in immature wheat endosperm (Don et al., 2003). If this is true, the PBs and glutenin particles should exhibit similarities at different levels: subunit composition, polymer composition, polymer size and polymer network properties.

In this study we have tested this hypothesis by comparing PBs from immature wheat with flour from mature grain of the same variety. The preparations from mature and immature grains were also compared for their ability to form a physical gel and for their composition of protein subunits and oligomers. Finally, their particle sizes were determined by laser diffraction and CLSM was used to study their compositions using specific stains for protein and free sulfhydryl groups.

Our results support the hypothesis that glutenin particles originate from the PBs. Additionally we suggest that further aggregation of individual PBs is required to generate the large particles observed in GMP.

2. Experimental

Wheat material used for protein body isolation was *Triticum aestivum* cv Cadenza of approximately 15 DAF (grown under controlled environment conditions at Rothamsted Research, Harpenden, UK). Flour was obtained by grinding mature dry grains from the same cultivar grown under the same conditions with an A11 Basic IKA Analytical Mill (IKA-WERKE GmbH & Co KG).

2.1. Protein body and protein body gel isolation

Endosperms were removed from all of the individual developing grain from 10 complete immature wheat spikes of *T. aestivum* cv Cadenza at approximately 15 DAF and chopped with a razor blade in 20 ml buffer 1 (20 mM, HEPES, pH7.6, 100 mM NaOAc, 5 mM $MgCl_2$). The homogenate was filtered through four layers of cheese cloth, pre-wetted with buffer 1. The residue was washed with 10 ml buffer 1 and the filtered homogenate was layered on top of a 1.75 M sucrose cushion in buffer 1 and centrifuged at 500*g* for 2 min at 10 °C. The material from the top of the cushion was collected and resuspended in 20 mM HEPES, pH7.6, 100 mM NaOAc, 5 mM EDTA, and 0.25 M sucrose, to a total volume of 10 ml. A two-step Percoll density gradient was prepared: a 1.13 g/ml and a 1.08 g/ml. The suspension was layered onto the two-step Percoll gradients and centrifuged at 7100*g* at 10 °C for 60 min. The PBs were collected in 0.5 ml volume from the surface of the 1.13 g/ml Percoll layer. The same volume of demineralised water was added to the PBs and the mixture vortexed briefly and then centrifuged for 2 min at 16,000*g*. The supernatant was removed and the pellet was resuspended in a total of 1 ml water. This was vortexed and centrifuged as before. Finally, the PB pellet was resuspended in an appropriate volume of demineralised water for immediate use. Further details are provided elsewhere (Davy et al., 2000).

SDS-insoluble protein was isolated by dissolving the PB pellet in 1.5% (w/v) SDS and centrifugation for 10 min at 16,000*g* at room temperature. The supernatant containing SDS-soluble proteins was decanted and kept for later analysis while the SDS-insoluble protein was recovered as a gel (PB-gel).

2.2. Isolation of SDS-insoluble (GMP) and SDS-soluble wheat protein from mature grain

0.5 g Flour of cv Cadenza was dissolved in 10 ml 1.5% (w/v) SDS. Ultracentrifugation was performed at 78,000*g* at 20 °C for 30 min (Centrikon T-2060, Kontron Instruments, USA). After ultracentrifugation, GMP was observed as a gel on top of a starch layer. The supernatant containing SDS-soluble protein was decanted and retained while the gel layer (GMP) was collected.

2.3. SE-HPLC analysis of SDS-soluble proteins (profible method)

Three replicates of 10 mg of PB pellet and 0.8 mg of flour were suspended in 1 ml 1% (w/v) SDS, 0.1 M sodium phosphate buffer (pH 6.9). Controlled ultrasonication with a sonicator (Vibra Cell 72434, Bioblock, Illkirch, France) delivering ultrasonic vibrations at 20 kHz and equipped with a 3 mm diameter tip probe was performed for 2 min without interruption (no overheating was observed), prior to separation by size-exclusion HPLC using a TSKgel G 4000 SW column (7.5 mm × 30 cm resolving column, Sigma Cat. No. 805790 and a 7.5 mm × 7.5 cm guard column, Sigma Cat. No. 805371). Further details are provided elsewhere (Dachkevitch and Autran, 1989; Millar, 2003; Morel et al., 2000).

2.4. SE-FPLC chromatography of SDS-soluble proteins

Size-exclusion FPLC was performed using a Superose 6 HR 10/30 column (GE Healthcare, UK) pre-equilibrated with 1% (w/v) SDS in 0.1 M sodium phosphate buffer (pH 7). Three replicates of aliquots (0.1 ml) of SDS-soluble protein from flour and from PBs were loaded on the column without prior sonication and fractionated using a flow rate of 0.3 ml/min. Eluate fractions of 0.5 ml were collected for SDS-PAGE analysis. The elution pattern was monitored at 280 nm.

2.5. SDS-PAGE gel electrophoresis

Fractions from SE-FPLC were analysed on a Multiphor II horizontal SDS electrophoresis system using ExcelGel SDS gradient 8–18 gels (GE Healthcare, UK). Proteins were precipitated using 10% (w/v) trichloroacetic acid (TCA) and after washing with ice-cold acetone, the air dried pellets were dissolved in sample buffer (2% (w/v) SDS, 62.5 mM Tris–HCl pH 6.2, 10% (w/v) glycerol, 0.01 mg/ml bromophenol blue) with and without 100 mM DTT as a reducing agent. Proteins were visualized using silver staining (Rabilloud et al., 1988). Protein body gel and fresh GMP fractions were also analysed on an ExcelGel SDS gradient 8–18 (GE Healthcare, UK) without prior precipitation.

2.6. Protein identification using nano-liquid chromatography – mass spectrometry

The bands of interest were excised from the SDS-PAGE gel and sliced into 1 mm^3 pieces. Gel pieces were processed essentially according to Shevchenko et al. (1996). Tryptic digests were then analysed by one-dimensional LC–MS using an Ettan™ MDLC system